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# Binding of extracellular matrix molecules by probiotic bacteria

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## ABSTRACT

I. ŠTYRIAK, R. NEMCOVÁ, Y.-H. CHANG AND Å. LJUNGH. 2003.

**Aims:** The aim of this study was to investigate extracellular matrix (ECM) and mucin binding of selected bacterial isolates with probiotic features in comparison with commercially used probiotic bacteria.

**Methods and Results:** ECM molecules were immobilized in microtitre plates (mucin and fetuin) or on the surface of latex beads. Porcine mucin was bound by all 13 probiotic strains tested with important inter-strain differences; however, fetuin binding was similar (weak) for all 14 strains tested. Strongly positive (three) binding of bovine fibrinogen was expressed by strains from fermented food (*Lactobacillus rhamnosus* GG, *L. casei* Shirota and *L. johnsonii* La1) as well as by *L. casei* L.c., *Lactobacillus* sp. 2I3 and by *L. plantarum* LP. The other strains expressed moderate (2) or weakly positive (1) binding of bovine fibrinogen. Strongly positive (3) binding of porcine fibronectin was observed only with two strains; however, all other strains also bound this molecule. Bovine lactoferrin was bound to a higher extent than transferrins.

**Significance and Impact of the Study:** Some animal strains (at least *L. casei* L.c. and *Lactobacillus* sp. 2I3) are comparable with the commercially used strains with respect to their ECM binding ability. As this feature is important for probiotic bacteria to be able to colonize intestine, these strains should be considered for their wider use in fermented feed (or probiotic preparations) for animals.

**Keywords:** binding, extracellular matrix, lactobacilli, mucin, probiotics.

## INTRODUCTION

During the past two decades probiotic micro-organisms have been increasingly included in various types of food products, especially in fermented milks (Saarela *et al.* 2000). However, there is also an increasing scientific and commercial interest in using beneficial (probiotic) micro-organisms to enhance animal health and feed conversion (Francisco *et al.* 1995; Chang *et al.* 2001). Probiotics have been defined in many different ways; however, the most common definition currently used is that of Fuller (1989): 'live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance.' A potentially successful probiotic strain is expected to have

several desirable properties in order to be able to exert its beneficial effects (Ouwehand *et al.* 1999). These effects are considered to include the prevention of gastrointestinal infections (Nemcová *et al.* 1998; Alvarez-Olmos and Oberhelman 2001), enhance immune response (Kimura *et al.* 1997; Vitini *et al.* 2000), and to exert antimutagenic as well as anticarcinogenic activity (Fuller and Gibson 1997; Parodi 1999). Many selection criteria have been considered to be relevant for any potential probiotic micro-organism. Successful probiotic bacteria should be able to colonize the mucosal surfaces, at least temporarily, and to prevent the attachment of pathogens such as *Escherichia coli* (Lee *et al.* 2000), *Helicobacter pylori* (Mukai *et al.* 2002) and other intestinal or food-borne pathogens (Todoriki *et al.* 2001).

The extracellular matrix (ECM) is a stable macromolecular structure underlying epithelial and endothelial cells and surrounding connective tissue cells (Westerlund and

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Korhonen 1993). Molecules of the ECM such as collagen-I and fibronectin can be shed into the mucus from the epithelium. Damaged host mucosae expose the ECM and this allows microbial colonization and infection. Adherence of pathogens to ECM of various host tissues has been investigated thoroughly, demonstrating the importance of these interactions in the establishment of many infections (Westerlund and Korhonen 1993; Ljungh and Wadström 1995). Moreover, ECM binding ability has been shown to be expressed by several pathogenic bacteria and to promote bacterial virulence (Lowrance *et al.* 1990; Patti *et al.* 1994; Hienz *et al.* 1996). Selected probiotic bacterial strains should be able to compete with pathogens for the same receptors and to occupy their potential binding sites in the gut (Neesser *et al.* 2000) including collagen-I (Štyriak *et al.* 1999a; Lorca *et al.* 2002) and fibronectin (Lorca *et al.* 2002).

The strains selected for our study were investigated for binding of seven selected glycoproteins in comparison with the probiotic strains, which are commercially used in probiotic food and preparations.

## MATERIALS AND METHODS

### Sources and cultivation of strains

Fourteen strains (11 strains of *Lactobacillus* spp., one *E. coli* and two *Enterococcus faecium* strains) with suitable probiotic properties were used in this study. Five *Lactobacillus* spp. strains (*L. paracasei* L81, *L. fermentum* L435, *Lactobacillus* sp. L457, L349 and L428) were originally isolated from the jejunum and ileum of piglets, *L. casei* subsp. *pseudoplantarum* L.c. from the intestine of a calf, *Lactobacillus* sp. 2I3 from a hen's intestine, *L. plantarum* LP from the silage preparation Labacsil® (SANO GmbH, Loiching, Germany) and three lactobacilli were from human probiotic food products (*L. rhamnosus* GG, *L. casei* Shirota and *L. johnsonii* La1) produced by Valio Dairy (Helsinki, Finland), Yakult (Tokyo, Japan), and Nestlé (Lausanne, Switzerland), respectively. *Escherichia coli* strain 083, originally isolated from a pig, is the component of the preparation Colinfant® (DYNTEC, Terezin, Czech Republic) for infant children. *Enterococcus faecium* EF was obtained from the silage preparation Labacsil® and *E. faecium* M-74, originally isolated from a child, was isolated from a probiotic preparation from Medipharm AB (Kågeröd, Sweden). Lactobacilli and enterococci were grown overnight in Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) and the *E. coli* strain in LB broth at 37°C. *Helicobacter pylori* CCUG 17874 was used as a positive control for fetuin and mucin binding. It was cultivated on GAB-CAMP agar (blood agar supplemented with 5% saponin lysed equine erythrocytes) under microaerophilic conditions (5% O<sub>2</sub>, 10% CO<sub>2</sub>, 85% N<sub>2</sub>) at 37°C for 2–3 days. *Staphylococcus aureus* Cowan1

strain was used as a positive control for the binding of the other ECM molecules. *Staph. saprophyticus* TW111 and *Streptococcus bovis* AO 24/85 were used as negative controls.

### Chemicals

Human holo-transferrin (iron-saturated), bovine apo-transferrin (iron-poor), mucin type III (partially purified) from porcine stomach, and fetuin type III from foetal calf serum were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Citric acid and glycine were purchased from Merck AG (Darmstadt, Germany). Porcine plasma fibronectin was purchased from BioInvent International AB (Lund, Sweden), bovine serum fibrinogen from Behring Diagnostics (La Jolla, CA, USA), merthiolate from Kabi AB (Stockholm, Sweden), crystal violet from Loba (Fischamend, Austria), Difco latex beads (0.81 µm diameter) from Difco Laboratories, and Nunc-Immuno microtitre plates with Maxi Sorp surface (96 wells) from Nunc International (Roskilde, Denmark). Bovine lactoferrin was kindly supplied by U. Svensson from the Swedish Dairies' Association Laboratory at Ideon (Lund, Sweden). All buffers and chemicals were of analytical grade.

### Adsorption of proteins to latex beads

Proteins were adsorbed to the Difco latex beads (0.81 µm diameter) by electrostatic and hydrophobic interactions as described previously (Naidu *et al.* 1988; Štyriak *et al.* 1999b).

### Particle agglutination assay (PAA)

The protein-coated latex beads (15 µl) were mixed on a glass slide with an equal volume of a bacterial cell suspension of 10<sup>10</sup> CFU ml<sup>-1</sup>. These two drops were gently mixed and the agglutination reaction was scored after 2 min as a PAA value from strongly positive (3) to weakly positive (1) or negative (0) as previously described (Naidu *et al.* 1988; Štyriak *et al.* 1999b). All strains were tested for autoaggregation by mixing one drop of a bacterial suspension with one drop of potassium phosphate buffer.

### Particle agglutination inhibition assay

Inhibitory effects were studied by preincubation of 100 µl of a protein with an equal volume of a bacterial suspension for 1 h at room temperature. After washing, the bacterial suspensions were mixed with the same protein-coated latex beads, and PAA values were scored as in the standard PAA.

### Microtitre plate binding assays

Bacterial binding of mucin and fetuin at a concentration of 100 µg ml<sup>-1</sup> was examined in Nunc-Immuno microtitre

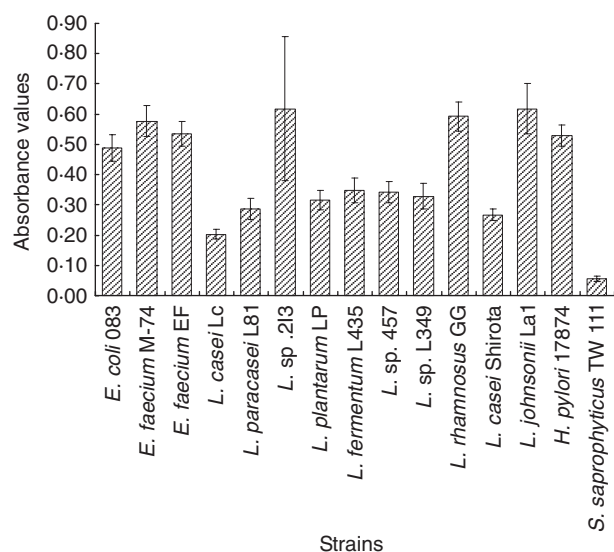
96-well plates as described previously (Štyriak and Ljungh 2003). The average of eight absorbance values, determined by a multiscan enzyme-linked immunosorbent assay reader, were calculated. Each batch of assays also included control strains with known protein binding levels as well as blank wells. *Helicobacter pylori* CCUG 17874 strain was used as a positive control, *Staph. saprophyticus* TW111 and *Strep. bovis* AO 24/85 (Štyriak *et al.* 1999b) were used as negative controls. Strains were classified as strongly adherent ( $A_{570\text{ nm}} > 0.3$ ), weakly adherent ( $0.1 \leq A_{570\text{ nm}} \leq 0.3$ ), or nonadherent ( $A_{570\text{ nm}} < 0.1$ ) as described previously (Štyriak and Ljungh 2003).

### Statistical analysis

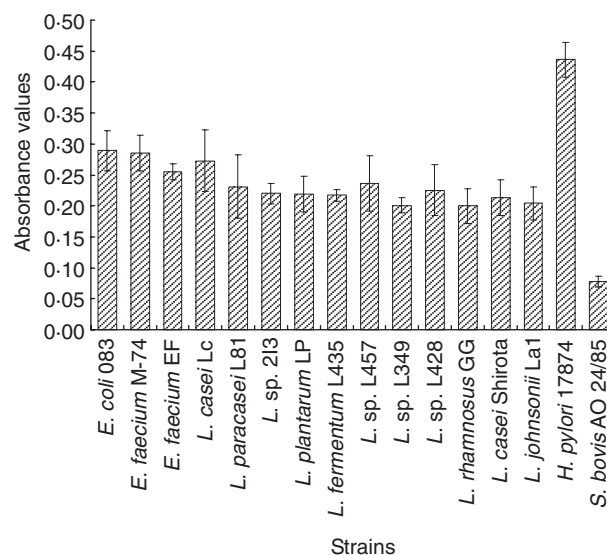
Statistical evaluation of the results was performed by one-way ANOVA. Significance of differences between mean values was calculated by Tukey's test.

## RESULTS

Immobilized porcine mucin in wells of microtitre plates was bound by all 13 probiotic strains tested (Fig. 1). By using the criteria given in Materials and methods, 10 strains were classified as strongly adherent and three as weakly adherent. However, inter-strain differences in mucin binding were relatively high. However, fetuin binding in microtitre plates was similar ( $A_{570\text{ nm}}$  values between 0.200 and 0.289) for the 14 strains tested (Fig. 2), i.e. all these strains bound fetuin weakly. Concerning the binding of ECM molecules tested by PAA, many inter-strain differences were shown (Table 1). Strongly positive (3) binding of bovine fibrinogen



**Fig. 1** Porcine mucin binding by probiotic strains. The values are expressed as mean  $\pm$  S.D. of eight absorbance ( $A_{570\text{ nm}}$ ) measurements



**Fig. 2** Fetuin binding by probiotic strains. The values are expressed as mean  $\pm$  S.D. of eight absorbance ( $A_{570\text{ nm}}$ ) measurements

was expressed not only by human commercial strains (*L. rhamnosus* GG, *L. casei* Shirota and *L. johnsonii* La1) but also by some other lactobacilli (*L. casei* subsp. *pseudoplantarum* L.c. from the intestine of a calf, *Lactobacillus* sp. 213 from a hen's intestine and *L. plantarum* LP from the silage preparation Labacsil). *Lactobacillus* sp. L349 expressed moderate (2) binding and the rest of the lactobacilli together with *E. faecium* EF showed weakly

**Table 1** Binding of five extracellular matrix molecules by strains with probiotic properties tested by particle agglutination assay (PAA)

Strain	BFg	PFn	BLf	Apo	Holo
<i>E. coli</i> 083	0	1	1	0	1
<i>E. faecium</i> M-74	0	1	0	0	0
<i>E. faecium</i> EF	1	2	1	0	1
<i>L. casei</i> L.c.	3	1	3	1	1
<i>L. paracasei</i> L81	1	1	1	0	0
<i>Lactobacillus</i> sp. 213	3	2	3	0	1
<i>L. plantarum</i> LP	3	3	3	1	2
<i>L. fermentum</i> L435	1	1	0	0	1
<i>Lactobacillus</i> sp. L457	0	1	0	0	0
<i>Lactobacillus</i> sp. L349	2	1	0	0	0
<i>L. rhamnosus</i> GG	3	2	1	1	0
<i>L. casei</i> Shirota	3	3	1	0	2
<i>L. johnsonii</i> La1	3	2	1	1	0
<i>S. aureus</i> COWAN 1	3	3	3	3	3
<i>S. bovis</i> AO 24/85	0	0	0	0	0

BFg, bovine fibrinogen; PFn, porcine fibronectin; BLf, bovine lactoferrin; Apo, bovine apo-transferrin; Holo, human holo-transferrin. The PAA values are expressed numerically as negative (0) weakly positive (1), moderate (2), and strongly positive (3) agglutination reaction.

positive (1) binding of bovine fibrinogen. Strongly positive (3) binding of porcine fibronectin was observed only with two strains (*L. casei* Shirota and *L. plantarum* LP); however, all other strains tested also bound this molecule (PAA values 1 or 2). Bovine lactoferrin was bound strongly (3) by the same three strains (*L. casei* subsp. *pseudopiantarum* L.c., *Lactobacillus* sp. 2I3 and *L. plantarum* LP) as bovine fibrinogen. Human lactobacilli expressed only weak (1) binding of this molecule. Many strains did not bind transferrins, especially bovine apo-transferrin. Those that did bind showed only weak (1) or moderate (2) binding. None of the enterococcal or *E. coli* 083 strains expressed strong binding of any ECM molecule, only mucin was bound by them strongly.

Some animal strains (at least *L. casei* L.c. and *Lactobacillus* sp. 2I3) are comparable with the commercially used strains with respect to their ECM binding ability.

## DISCUSSION

It is becoming increasingly recognized that probiotic micro-organisms with beneficial effects for their hosts may offer an alternative to conventional antimicrobials in the treatment and prevention of enteric infections. This has become more important in recent years when increasing antibiotic resistance may soon render conventional therapy inadequate for many infections. It seems that adherence and expression of some antagonistic activity against pathogens, especially against their adhesion, are amongst the most important features for probiotic micro-organisms (Reid 1999). Binding of shed ECM molecules provides one mechanism of intestinal colonization, and indeed collagen binding as well as fibronectin binding have been proposed to mediate such adhesion (Sillanpää *et al.* 2000; Lorca *et al.* 2002). Moreover, the mucin layer is the first place of contact between the host and micro-organisms (Cohen *et al.* 1983) and this is why probiotic bacteria expressing good mucin binding ability may be able to colonize and possibly prevent subsequent enteric infections.

This study was undertaken in order to compare some strains with beneficial effects for their hosts (Saarela *et al.* 2000), which are commercially used in fermented food as well as strains with useful fermentation properties (used in silage preparations) with selected animal strains. We found that some animal strains (*Lactobacillus casei* L.c. and *Lactobacillus* sp. 2I3) are comparable with the commercial strains with respect to their ECM binding ability. These strains should be tested further for potential use in animal feed products.

In previous studies (Štyriak *et al.* 2001; Štyriak and Nemcová 2003), lactobacilli were cultured on solid (Rogosa agar) as well as in liquid medium (MRS broth). For the present study, the strains were cultivated only in MRS broth because probiotic preparations are preferably applied in

liquid form (as fermented food or feed). *Lactobacillus paracasei* L81 strain bound fetuin to a higher extent in the present study than the previous (Štyriak *et al.* 2001; Štyriak and Nemcová 2003), whereas porcine fibronectin was bound to a lower extent. *Lactobacillus casei* L.c. displayed similar results as previously described (Štyriak *et al.* 2001; Štyriak and Nemcová 2003) with the exception of bovine fibrinogen and bovine lactoferrin binding. These differences suggest that not only the medium but also other conditions can affect the expression of ECM binding structures on the bacterial surface. In this context, it is interesting to note that *Lactobacillus* sp. strain 2I3 from a hen's intestine displayed autoaggregation after washing in PBS; however, autoaggregating cells (forming a precipitate in a clear solution) were not observed after its washing in potassium phosphate. It suggests that Na<sup>+</sup> ions mediate aggregation at least in this strain. However, autoaggregating strains express profound cell surface hydrophobicity, and this may also enhance intestinal colonization.

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